Studies on the Properties of Cow's-Milk Tributyrinases and their Interaction with Milk Proteins

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1. The tributyrinases in milk are mainly associated with casein micelles. Dilution or addition of sodium chloride increases the enzyme activity, probably by dissociating the micelle–tributyrinase complexes. 2. Tributyrinase activities of milks activated by dilution and sodium chloride addition were in the range 0·2–1·7 μequiv. of acid liberated/ml. of milk/min. from tributyrin emulsion at pH8·5 and 25°. The enzymes have a bivalent-cation requirement for full activity and are rather unstable when separated from casein. 3. Ultracentrifugation of skim milks containing sodium chloride (0·75M) gave preparations low in casein but containing about 70% of the milk tributyrinases. The tributyrinases in such preparations appear to be bound in complexes of molecular weight about 350000. Dilution may result in dissociation to give the free enzymes. 4. Pancreatic lipase also formed complexes with casein micelles, but wheat-germ esterase, xanthine oxidase, milk alkaline phosphatase and other enzymes did not.

Most of the tributyrinase activity in cow's milk is sedimented with the casein micelles by high-speed centrifugation, and this close association with casein has greatly hindered its investigation. The preparation of milk tributyrinase solutions containing only small amounts of casein (Downey & Andrews, 1965*a*) opened the way to further investigations. Gel-filtration experiments indicated the presence in such preparations of at least four enzymes that hydrolysed emulsified tributyrin, and enabled us to make tentative estimates of their molecular weights. We now describe a gel-filtration study of the interaction between milk tributyrinases and casein micelles. Some of the results have already been reported briefly (Downey & Andrews, 1965*b*).

MATERIALS AND METHODS

Purified α-, β- and κ-casein were kindly provided by Dr G. C. Cheeseman, and purified milk xanthine oxidase and milk alkaline phosphatase by Dr R. C. Bray and Dr R. L. J. Lyster respectively. Crude pig pancreatic lipase was expressed from fresh tissue (Downey & Andrews, 1965*a*), and wheat-germ esterase was an aqueous solution (10mg./ml.) of a freeze-dried preparation (L. Light and Co. Ltd., Colnbrook, Bucks.).

Cow's-milk preparations. Skim milks were prepared from uncooled raw milks about 1hr. after their collection from individual Friesian cows by using a Lister cream separator, and were stored in the dark at 2°. Supernatant A was prepared by centrifuging skim milk in a Spinco model L ultracentrifuge (rotor 30) at 80000*g* for 1hr. at 2°, and gently decanting the solution. Some slowly sedimented casein was carried over with the solution. Supernatant B was also prepared from skim milk but centrifugation was extended to 2hr. at 80000*g* and the clear supernatant was collected through a hole pierced in the Lusteroid centrifuge tube about 1cm. above the casein pellet.

NaCl-supernatants A and B were prepared by similar procedures from skim milk to which NaCl (0·75M final concn.) had first been added. The $E_{340m\mu}$ of NaCl-supernatant B preparations was taken as a measure of their casein-micelle content, and any with $E_{340m\mu}$ greater than 0·2 were discarded. A preparation with a very low casein content was obtained from NaCl-supernatant A by the calcium precipitation method of Bohren & Wenner (1961).

Clarifier slime (yield approx. 10g. wet wt. in each case) was obtained from four 100l. lots of raw bulk milk by passing it through an Alfa-Laval separator fitted with the standard clarifier modification.

Enzyme assays. Assays for milk tributyrinases and pancreatic lipase were performed at pH8·5 and 25° with 2ml. of tributyrin emulsion as substrate, and wheat-germ esterase was estimated at pH7·5 with 1ml. of triacetin solution.

Tributyrin, triacetin and gum acacia were obtained from British Drug Houses Ltd., Poole, Dorset. Tributyrin emulsion was prepared from the triglyceride (10ml.), gum acacia (10g.) and ice–water (80ml.) as described by Downey & Andrews (1965*a*). The pH of the resultant emulsion was adjusted to 8·5 with N-NaOH, and water added to a final volume of 100ml. Triacetin solution was prepared by dissolving the triglyceride (1ml.) in water (100ml.) and adjusting the pH to 7·5.

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Acid release from tributyrin and triacetin was measured by continuous titration with a Radiometer titrator type TTT1c coupled to a Radiometer titrigraph type SBR2c (Radiometer, Copenhagen, Denmark). The assay mixture (2–12ml.), contained in a water-jacketed cell, was continuously stirred and bubbled with CO_2-free N_2. We have previously described the assay method in detail (Downey & Andrews, 1965*a*), but experience shows that the following additional points are important The outlet from the microsyringe of the titrator was in contact with the bulb of the glass electrode, to ensure immediate detection by the pH-meter of the smallest additions of alkali. Static electricity rendered the pH-meter readings unsteady and caused irregular titration in measurements at maximum sensitivity, unless the dial glass of the pH-meter was coated with glycerol, NaCl was added to the water circulating through the reaction jacket and the complete apparatus was earthed to a stainless-steel sheet on which it stood. Tributyrinase in solutions of low ionic strength (e.g. dialysed solutions) was assayed satisfactorily only if salt (1ml. of 0·1M-NaCl) was added to the assay mixture.

The volume of enzyme preparation used, and hence the total assay volume, varied in different experiments. Nevertheless, rates of acid production were linear with time and proportional to the amount of enzyme added. Each assay was run for at least 5min. One unit of activity is taken as the amount of enzyme liberating 1μequiv. of acid from the substrate/min. With 1mN-NaOH as titrant, the method was used to measure 10–70milliunits of enzyme activity, allowing tributyrinase assays on as little as 0·02ml. of milk. To assay the activity of larger milk samples, 10mN- or 100mN-NaOH was used. When the effect of NaCl concentration on milk tributyrinase activity was being investigated, NaCl was added to the assay mixture to give a final concentration similar to that in the milk sample.

Gel filtration. Columns (50cm.×2·4cm. diam.) of Sephadex G-200 [Pharmacia (Great Britain) Ltd., London] were packed in the cold with the swollen gels as described by Flodin (1961) or Andrews (1965) and equilibrated with salt solutions made up in redistilled water. Effluent was collected in 5ml. fractions by using a collector fitted with a siphon. All columns were run at 0–5° and flow rates were 10–15ml./hr. The columns were calibrated for estimating the molecular weights of proteins as described by Andrews (1964, 1965), with as standards the proteins of known molecular weights listed by Downey & Andrews (1965*a*). Purified *Escherichia coli* phosphatase, rabbit-muscle lactate dehydrogenase and *E. coli* β-galactosidase (10–20μg. of each) were included in some column runs as markers. They were assayed as described by Garen & Levinthal (1960), Kornberg (1955) and Wallenfels (1962) respectively.

Dialysis. Milk preparations (50ml.) contained in Visking 36/32in. tubing (Hudes Merchandising Corporation Ltd., London) were dialysed at 2° against distilled water or salt solution (5l.) with stirring.

RESULTS

Tributyrinase activity of milk and skim milk

Estimation of the tributyrinase activity in milk was combined with an investigation of the conditions necessary to elicit maximum activity.

Assays on 10ml. samples of whole milks and of the corresponding skim milks indicated the presence of 30–50% more tributyrinase activity/ml. in the skim milks than in the whole milks. Up to 15% of the activity of whole milk was detected when no substrate was added, but none was detected in skim milk. When assays were performed on 0·05ml. samples of skim milk, 40–60% more tributyrinase activity/ml. of sample was detected than when assays were performed on 10ml. samples.

Addition of sodium chloride to skim milk resulted in an increase in tributyrinase activity. Maximum increases occurred at salt concentrations of 0·5–0·75M, and for different milks were in the range 40–100% when 10ml. samples were assayed but were only 5–20% for the same milks when 0·05ml. samples were assayed. Inhibition occurred at salt concentrations above 0·75M. The activity was again equal to its original value at about 1·3M and lower still at higher concentrations.

Tributyrinase activities of fresh whole milks from 61 Friesian cows were in the range 0·2–1·7units/ml., with a mean of 0·88unit/ml., when 0·2ml. samples were assayed in the presence of 0·6M-sodium chloride.

Tributyrinase activity of milk fractions

Ultracentrifugation of skim milk sedimented most of the casein and 70% of the tributyrinase (Table 1). Assays with 0·05ml. or 10ml. samples of the supernatant solution (supernatant A) gave identical values for tributyrinase activity/ml., and no increase in activity was detected when samples were assayed in the presence of 0·75M-sodium chloride. Ultracentrifugation in the presence of 0·75M-sodium chloride yielded supernatants (sodium chloride-supernatants A and B) that contained considerably more tributyrinase than those prepared in the absence of sodium chloride, and assays on 0·05ml. or 10ml. samples of these again gave identical values for tributyrinase activity/ml.

Stability and pH *optimum of milk tributyrinase*

The tributyrinase activity of whole milks decreased by 10–22%, and that of the corresponding skim milks by 30–50%, during storage for 7 days in the dark at 2°. The activities of preparations of sodium chloride-supernatants A and B fell by 5–10% and 15–20% respectively during 1 day in the dark at 2°.

The thermal stability of skim-milk tributyrinase was tested by heating skim milk at 25°, 37° and 60°. Samples from the 25° and 60° incubations were assayed at 25°, and those from the 37° incubation at 37°. Initial activity at 37° was 53% higher than that at 25°. Only about 9% of the activity was lost in

Table 1. *Analytical results for skim milk and high-speed supernatants prepared therefrom*

Preparation of the high-speed supernatants is described in the Materials and Methods section. Tributyrinase activity was estimated on 0·05ml. samples in each case. Total N and casein N were determined by the method of Rowland (1938), and sialic acid by the thiobarbituric acid method of Warren (1959).

	Tributyrinase activity (unit/ml.)	Total N (mg./ml.)	Casein N (mg./ml.)	Sialic acid (μg./ml.)
Skim milk	—	4·88 (100%)	3·85 (100%)	96·0 (100%)
Skim milk + 0·75M-NaCl	0·82 (100%)	4·90	3·86	—
Supernatant A	0·24 (29%)	1·29 (26%)	0·16 (4·2%)	—
Supernatant B	0·22 (27%)	1·23 (25%)	0·14 (3·5%)	—
NaCl-supernatant A	0·56 (68%)	1·59 (32%)	0·42 (11%)	—
NaCl-supernatant B	0·53 (65%)	1·27 (26%)	0·14 (3·5%)	1·52 (1·5%)

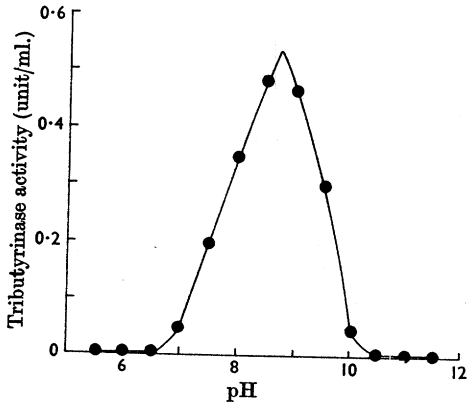

Fig. 1. Optimum pH of skim-milk tributyrinase. Assays were performed on 10ml. samples.

6hr. at 25°, whereas 83% was lost in 4hr. at 37° and almost complete inactivation occurred in 4min. at 60°.

Skim-milk tributyrinase showed maximum activity at pH 8·7 when assayed in the range pH 5·5–11·5 (Fig. 1). Initial pH adjustment of the assay mixtures was with 0·1N-hydrochloric acid or 0·1N-sodium hydroxide as necessary.

Effect of dialysis and EDTA addition on the activity of milk tributyrinase

Skim milk lost no tributyrinase activity during dialysis against water, but dialysis of sodium chloride-supernatant A preparations from six different skim milks against water, 0·75M-sodium chloride or 0·75M-potassium chloride for 4–5hr. resulted in the loss of 10–55% of their tributyrinase activity, the losses being reproducible but varying from milk to milk. Dialysis for another 15hr., with two changes of the water, effected no further decrease.

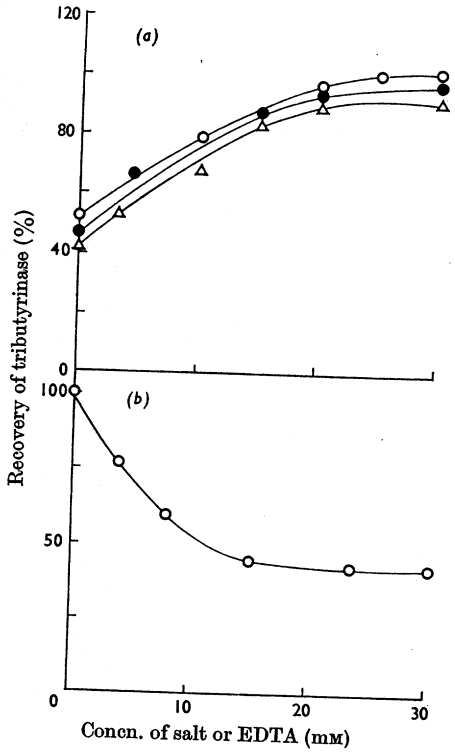

Fig. 2. (*a*) Effect of dialysis for 18hr. against $MgCl_2$ (○), $MnCl_2$ (●) or $CaCl_2$ (△) on the tributyrinase activity of NaCl-supernatant A, prepared from skim milk as described in the text. (*b*) Effect of addition of EDTA on the tributyrinase activity in skim milk containing NaCl (0·75M). Assays were performed on 5ml. samples.

No activity was lost during dialysis against magnesium chloride, manganous chloride or calcium chloride (25mM in each case), but dialysis against progressively lower concentrations of these salts resulted in progressively greater losses of activity

(Fig. 2*a*). Addition of the salts (25mM final concentration) to preparations that had lost activity by dialysis against water failed to restore activity.

When skim milks containing sodium chloride (0·75M) were added to assay mixtures containing sodium chloride (0·75M) and EDTA, rates of acid production were linear for at least 10min., but less than in the absence of EDTA (Fig. 2*b*). The activity losses corresponded approximately to the losses by sodium chloride-supernatant A preparations from the same skim milks during dialysis against water.

As a further indication of a bivalent-ion requirement, recoveries of tributyrinase activity were much more variable and lower in gel-filtration experiments when 0·75M-sodium chloride was used as column eluent than when 25mM-magnesium chloride was also present.

Gel-filtration studies

Effect of sample size on the behaviour of milk tributyrinases. Samples of sodium chloride-supernatant A, prepared from one milk and ranging in size from 3ml. to 18ml., were submitted to gel filtration on a column equilibrated with 0·75M-sodium chloride containing magnesium chloride (25mM). The results are shown in Fig. 3.

In order of increasing elution volume, the five peaks in the protein elution diagram of Fig. 3(*e*) probably represent casein micelles, milk globulins plus casein aggregates, β-lactoglobulins, α-lactalbumin and low-molecular-weight material respectively (cf. Downey & Andrews, 1965*a*). The same identifications presumably apply to peaks in the other protein diagrams, although when larger samples were used the first two peaks were not resolved. Tributyrinase activity was eluted in similar positions whether 4ml. (Fig. 3*e*) or 3ml. samples were used, but it was eluted progressively earlier as the sample size was further increased, and appeared eventually as an asymmetric band of ill-defined peaks. Increasing the sample volume from 10ml. to 18ml. effected only a slight change in the elution volume of the main tributyrinase peak and in both cases it was eluted later than the casein-micelle peak, in a position corresponding to that of a 'typical' globular protein (Andrews, 1965) of molecular weight about 350000. Recoveries of tributyrinase ranged from 25% for the 3ml. sample to 70% for the 18ml. sample. The elution volumes of the enzyme markers were unaffected by sample size.

Changes in sample size had much less effect on the behaviour of tributyrinases in sodium chloride-supernatant B than they did on their behaviour in sodium chloride-supernatant A. The tributyrinases in 10ml. of sodium chloride-supernatant B were still eluted in the same positions as they were when applied to the columns in 3ml. or 4ml. of sodium chloride-supernatant A, and increasing the sample volume to 30ml. resulted in only small shifts in their elution volumes towards the column void volume.

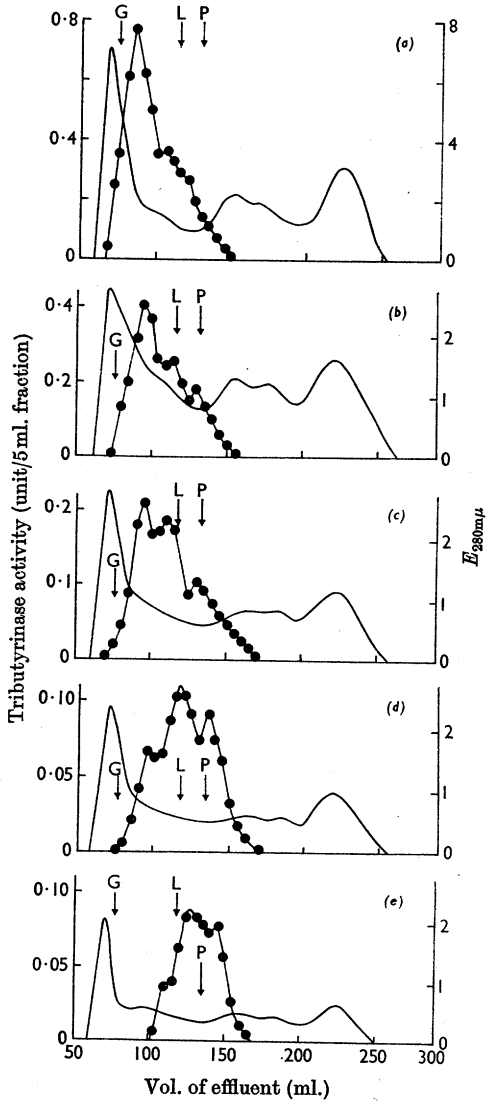

Fig. 3. Effect of sample size on the gel-filtration behaviour on Sephadex G-200 columns of the tributyrinases in NaCl-supernatant A (0·75M-NaCl), prepared from skim milk as described in the text. Elution was with 0·75M-NaCl containing $MgCl_2$ (25mM). Volumes of NaCl-supernatant A used were: (*a*) 18ml.; (*b*) 10ml.; (*c*) 6ml.; (*d*) 5ml.; (*e*) 4ml. ——, $E_{280m\mu}$; ●—●, tributyrinase activity. G, L and P (arrows) indicate the elution volumes of *E. coli* β-galactosidase, lactate dehydrogenase and *E. coli* phosphatase respectively, used as markers.

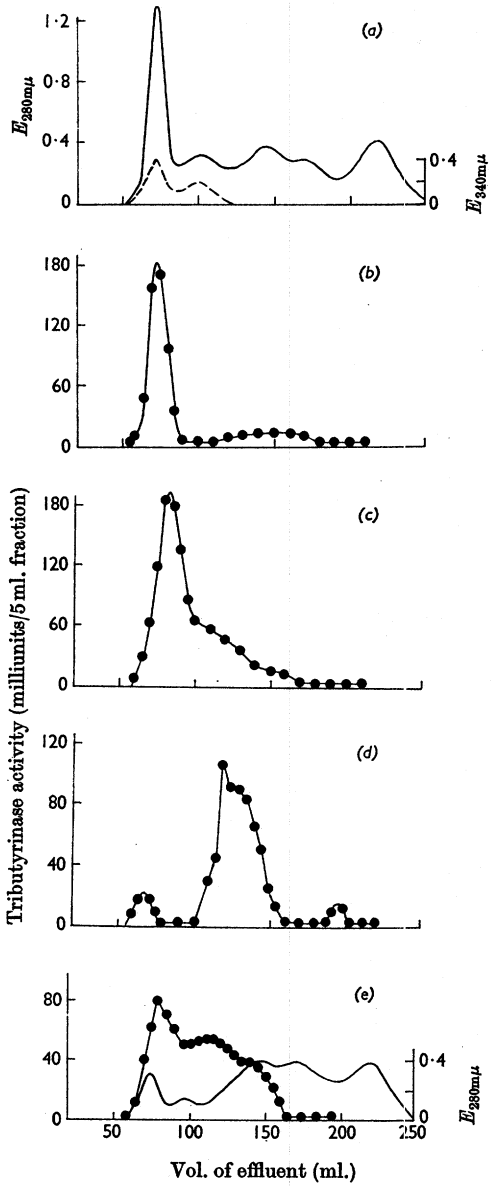

Fig. 4. Effect of NaCl concentration on the gel-filtration behaviour on Sephadex G-200 columns of the tributyrinases (●—●) in two NaCl-supernatant A preparations, obtained from skim milk as described in the text. One preparation is shown in (*a*)–(*d*) and the other in (*e*). Each run was performed with 4ml. of the preparation. Elution diagrams for protein (——, $E_{280m\mu}$) in each preparation are shown in (*a*) and (*e*). The presence of casein micelles and β-casein aggregates is shown in (*a*) by $E_{340m\mu}$ (– – – –) measured at 25° (the explanation is given in the text). Columns were equilibrated and eluted with salt solutions as follows: (*a*) and (*b*), 50mM-NaCl containing $MgCl_2$ (25mM); (*c*) and (*e*), 0·2M-NaCl containing $MgCl_2$ (25mM); (*d*), 0·75M-NaCl containing $MgCl_2$ (25mM).

Tributyrinase recoveries were about 35, 50 and 70% with 10ml., 20ml. and 30ml. samples of sodium chloride-supernatant B respectively.

Effect of ionic strength on the behaviour of tributyrinases. Sodium chloride-supernatant A preparations from two different milks were submitted to gel filtration on columns equilibrated with solutions containing various concentrations of sodium chloride and magnesium chloride (25mM). The results are shown in Fig. 4.

The interpretation of the protein elution diagrams in Figs. 4(*a*) and 4(*e*) is the same as for Fig. 3, but in addition the presence of β-casein aggregates (estimated mol.wt. approx. 200000) in the second peak (V_e approx. 100ml.; Fig. 4*a*) was indicated by the development of opalescence (measured by $E_{340m\mu}$) in this region when the temperature of fractions from the runs at lower ionic strengths rose to 25°. β-Casein is much more soluble in the cold than at 25° (Waugh, 1961). The non-appearance of opalescence in this region in fractions from runs in 0·75M-sodium chloride containing magnesium chloride (25mM) is presumably due to a greater solubility of β-casein under such conditions. The opalescence associated with the casein-micelle peak showed no change with temperature.

Most of the tributyrinase activity of each preparation was eluted with the casein micelles from columns equilibrated with 50mM-sodium chloride containing magnesium chloride (25mM) (Fig. 4*b*), whereas very little enzyme appeared in the casein region in the presence of 0·75M-sodium chloride containing magnesium chloride (25mM) (Fig. 4*d*) (cf. Downey & Andrews, 1965*a*). In 0·2M-sodium chloride containing magnesium chloride (25mM), tributyrinase was eluted partly in a peak that emerged 10ml. later than the casein-micelle peak and partly in a zone extending from this peak to the typical positions of the enzymes at high ionic strengths (Figs. 4*c* and 4*e*). The proportion of tributyrinase in the peak was greater in the preparation that, to judge from its protein elution diagram (Fig. 4*a*), contained the larger amount of casein micelles. Tributyrinase recoveries ranged from 70% in experiments at the lowest sodium chloride concentration to 40% in those at the highest. Changes in the ionic strength of the eluent had no effect on the elution volumes of the marker enzymes.

Effect of milk fractions on the behaviour of pancreatic lipase and wheat-germ esterase. When crude pig pancreatic lipase (3·2units) and wheat-germ esterase (1·5units) were separately submitted to gel filtration on a column equilibrated with 0·1M-sodium chloride, they were eluted in positions corresponding to molecular weights of 42000 and 51000 respectively (Fig. 5*a*; cf. Downey & Andrews, 1965*a*), with activity recoveries of 50 and 100%. They were both eluted in the same positions when

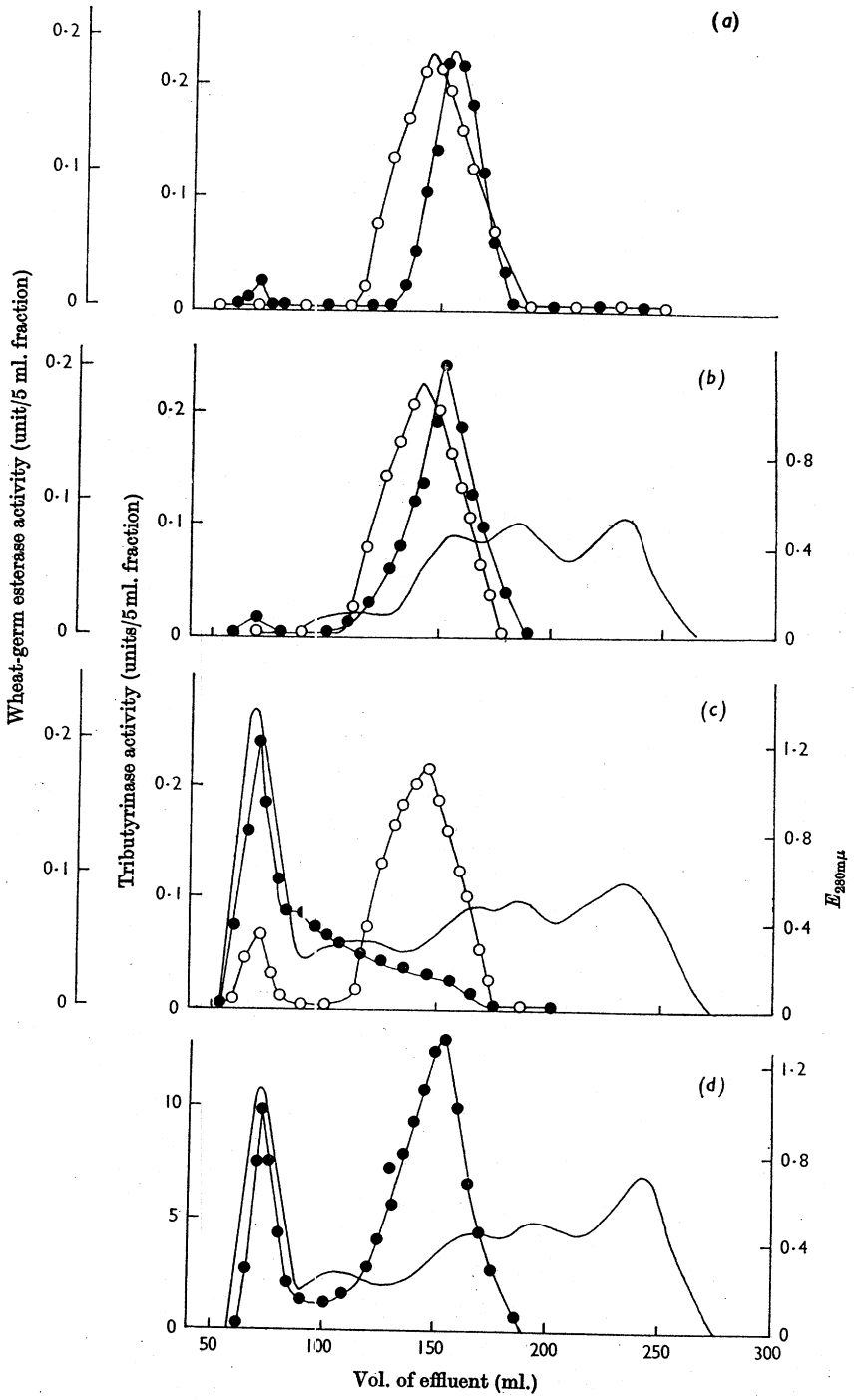

Fig. 5. Gel filtration on Sephadex G-200 of various amounts of pancreatic lipase (●—●) or wheat-germ esterase (○—○) (*a*) alone, (*b*) in the presence of 4 ml. of a casein-free NaCl-supernatant A preparation and (*c* and *d*) in the presence of 4 ml. of NaCl-supernatant A. The supernatant preparations were obtained from skim milk as described in the text. Elution was with 0·1 M-NaCl. The experiments with lipase and esterase were performed separately, and the results are superimposed here to facilitate comparison. ——, $E_{280m\mu}$.

mixed with casein-free sodium chloride-supernatant A (Bohren & Wenner, 1961) containing no milk-tributyrinase activity (Fig. 5*b*). The absence of casein micelles from this supernatant preparation is shown by the absence of a peak from the appropriate place in its protein elution diagram. Gel filtration in 0·1M-sodium chloride of pancreatic lipase (3·2units) mixed with 4ml. of sodium chloride-supernatant A containing casein micelles and some milk-tributyrinase activity (1·3units), however, resulted in about half the eluted lipase (tributyrinase activity) appearing with the casein-micelle peak, with the remainder trailing behind (Fig. 5*c*). The recovery of tributyrinase activity was low (2·1units), but more than the amount of milk tributyrinase in the original sodium chloride-supernatant A sample. Much more tributyrinase activity (25units) than that in the milk preparation appeared with the casein-micelle peak when a mixture of pancreatic lipase (143units) with 4ml. of sodium chloride-supernatant A was run under the same conditions, the remaining enzyme (110units) being eluted in the position of free pancreatic lipase (Fig. 5*d*). Wheat-germ esterase (1·5units), mixed with 4ml. of sodium chloride-supernatant A, which had no detectable activity towards triacetin solution, and run under the same conditions, was eluted mainly in the position of free enzyme, only about 0·15unit of activity being with the casein micelles (Fig. 5*c*).

Various amounts of xanthine oxidase, milk alkaline phosphatase and the marker enzymes showed no tendency to combine with the casein micelles of sodium chloride-supernatant A during gel filtration in 0·1M-sodium chloride.

Crude pig pancreatic lipase (1·0unit) was mixed with purified sodium α-, β- and κ-casein (5–10mg.) and each mixture submitted to gel filtration on a column equilibrated with 0·1M-sodium chloride. Both in the presence and absence of the lipase, the α- and κ-casein were eluted at the column void volume, whereas the β-casein was eluted later, in a position corresponding to that of a 'typical' globular protein of molecular weight about 130000. A very small amount of the lipase emerged at the column void volume even when it was run without added casein (cf. Fig. 5*a* and Downey & Andrews, 1965*a*), but otherwise it was well separated from the caseins, and in no case did the presence of casein alter its gel-filtration behaviour. However, some 'tailing' of the β- and κ-casein peaks in the presence of lipase indicated that proteolytic enzymes in the lipase preparations effected some degradation of these caseins.

Attempted preparation of lipase from clarifier slime. Attempts were made to isolate lipase from clarifier slime by the procedure of Chandan & Shahani (1963*a*). Tributyrinase activities in aqueous extracts of the slime ranged from 0·3 to 2·1 units/ml., but the activities of preparations obtained after acetone and ammonium sulphate fractionation of the extracts were insufficient for further experiments. Gel filtration of the slime extracts was impeded by their very high viscosities in the presence of 0·75M-sodium chloride containing magnesium chloride (25mM). Tributyrinase recovered in low yield (10–20%) from some runs with this eluent emerged from the column only with the casein-micelle peak.

DISCUSSION

General properties of milk tributyrinases. Our experiments confirm that most of the tributyrinase activity in milk is associated with the casein micelles (Sjöström, 1959; Jensen, 1964; Chandan & Shahani, 1964). The higher tributyrinase activity of skim milk as compared with that of whole milk is probably not an activation effect resulting from the process of cream separation because Mattick & Kay (1938) found the same thing occurred when the cream was allowed to rise of its own accord. A likely explanation is that the enzymes have kinetic properties and substrate affinities that result in the acid liberation from tributyrin alone being more rapid than that from a mixture of tributyrin and milk fat. Milk lipase does hydrolyse emulsified tributyrin at a faster rate than it hydrolyses milk-fat emulsion (Frankel & Tarassuk, 1956*a*,*b*).

The tributyrinases in skim milk are readily inactivated by heating and are unstable even at 37°. As they are more active at 37° than at 25° they are often assayed at the higher temperature, but our results indicate that this is unwise. Association with casein micelles evidently confers some stability on the enzymes, possibly by protecting thiol groups (Frankel & Tarassuk, 1959), for in supernatant preparations and in column effluents they are less stable than in skim milk and the instability increases as the casein content of the preparations decreases. For reasons that have not been determined, a total loss of tributyrinase activity occurred occasionally in gel-filtration column runs.

The pH optimum of milk lipase activity varies with the substrate, but is generally in the range pH 8–9 (Frankel & Tarassuk, 1956*a*). Our observations agree with this. With tributyrin emulsion as substrate, we did not observe any of the other pH optima reported for milk lipases (cf. Chandan & Shahani, 1964). Good agreement exists between our estimates of tributyrinase activities in cow's milk and those of Frankel & Tarassuk (1956*b*), and in both cases the assay conditions were probably optimum. Surprisingly, Dunkley & Smith (1951*a*,*b*) also found similar values, although they used tributyrin solution rather than tributyrin emulsion as substrate.

The results of dialysis experiments and of EDTA addition indicate a bivalent-cation requirement for full activity of milk tributyrinases. The maintenance of skim-milk tributyrinase activity during dialysis suggests in this case the continued availability to the enzymes of calcium, which, being part of the micelle structure, was not lost during dialysis. Ca^{2+} ions affect the activity of pancreatic lipase (Desnuelle, Naudet & Constantin, 1950; Borgström, 1954) and other lipases (Wood, 1959; Iwai, Tsujisaka & Fukumoto, 1964), but generally it is uncertain to what extent the effects are due to activation processes, to removal of liberated fatty acids or to enzyme stabilization.

Dissociation of casein micelle–tributyrinase complexes. Milk tributyrinases associated with casein micelles in skim milk are evidently not fully active, but both dilution and addition of sodium chloride are effective in stimulating or restoring their activity, apparently by dissociating the micelle–tributyrinase complexes. Neither process enhanced the activity of supernatant preparations that contained little or no tributyrinase associated with casein micelles. A greater separation of tributyrinase from casein during centrifugation in the presence of sodium chloride than in its absence is shown by a comparison of the casein and tributyrinase contents of supernatant B preparations from salted and unsalted skim milk (Table 1). Dilution of milk decreases the size of the casein micelles (Nitschman, 1949) and both dilution and addition of sodium chloride are believed to cause their partial dissolution (Hippel & Waugh, 1955; Zittle & Jasewicz, 1962). The data in Table 1 indicate, however, that, though the sedimentation rate of casein in skim milk may be decreased in the presence of sodium chloride, the amount of sedimentable casein is not significantly altered. The inhibition of milk tributyrinases by high concentrations of sodium chloride (cf. Willart & Sjöström, 1959) clearly limits its effectiveness for activation, but a combination of dilution and addition of sodium chloride seems likely to elicit the maximum activity of the enzymes. Dilute samples of skim milk were much less activated by sodium chloride than were concentrated samples.

The variations in enzyme recoveries in the gel-filtration experiments necessitate particular caution in the interpretation of results. Nevertheless, experiments with sodium chloride-supernatant A indicated that, even when the micelle–tributyrinase complexes were dissociated in 0·75M-sodium chloride, increases in sample size, with the attendant lessening in dilution during passage through the column, resulted in progressive decreases in the elution volumes of the tributyrinases and decreases in the separations between them. Even with the largest sample, however, the enzyme peak was eluted later than the casein-micelle peak. One interpretation is that in sodium chloride-supernatant A the tributyrinases are in complexes with molecular weights of about 350000, which are not appreciably sedimented in 1–2hr. at 80000*g*. Dilution induces dissociation of these complexes to give the free enzymes. Presumably the tributyrinases in unsalted skim milk that are not sedimented under similar conditions also exist in such complexes. Experiments with different preparations of sodium chloride-supernatants A and B suggested that the casein-micelle concentration in these preparations affected the dissociation of the non-sedimentable complexes, but this effect is not readily explained at present.

The tributyrinases in sodium chloride-supernatant A became associated with micelles when the salt concentration was sufficiently decreased. Pancreatic lipase similarly formed complexes with the casein micelles in sodium chloride-supernatant A, but wheat-germ esterase and various other enzymes showed little or no tendency to do so. At a low salt concentration pancreatic lipase behaved as free enzyme in the presence of a casein-free supernatant preparation.

Molecular weights of milk tributyrinases. The interaction between milk tributyrinases and other proteins clearly increases the uncertainty of molecular weights estimated for them by the gel-filtration method. Values of 62000, 75000 and 112000 given for three of the enzymes in a previous study (Downey & Andrews, 1965*a*) were based on their behaviour when applied to columns in 4ml. samples of sodium chloride-supernatant A. The present experiments on the effect of sample size on the behaviour of the tributyrinases in sodium chloride-supernatants A and B indicate that, in such samples, the interaction between tributyrinases and other proteins is decreased to a minimum. For this reason we think that our previous molecular-weight estimations were not seriously in error.

Possible identity of the milk fraction that binds tributyrinases. Skean & Overcast (1961) suggested that α-casein binds milk lipase, and Yaguchi, Tarassuk & Abe (1964) attributed this property to κ-casin, which is part of the α-casein fraction, because milk lipase activity was to some extent correlated with the amount of sialic acid in fractions from the ion-exchange chromatography of casein preparations. However, κ-casein may not be the only casein fraction that contains sialic acid (Cayen, Henneberry & Baker, 1962), and we found that almost all the sialic acid was removed from skim milk in the preparation of sodium chloride-supernatant B, which nevertheless contains a major part of the skim-milk tributyrinase (Table 1).

Some observations indicate that β-casein binds

lipase. Saito & Hashimoto (1963) found similarities in the behaviour of milk lipase and β-casein during ion-exchange chromatography, and Shahani & Chandan (1965) observed complex-formation between β-casein and clarifier-slime lipase in the ultracentrifuge. β-Casein aggregates in sodium chloride-supernatant A, detected in gel-filtration experiments, appeared to have a molecular weight of about 200000. Aggregates of purified β-casein were somewhat smaller, but those of α- and κ-casein were much larger. Thus stoicheiometric association of β-casein aggregates and milk tributyrinases with molecular weights of 120000 or less (Downey & Andrews, 1965*a*) seems more likely to give complexes with the gel-filtration behaviour of those in sodium chloride-supernatant A than does association between α- or κ-casein and the tributyrinases. The failure of pancreatic lipase to associate with sodium α-, β- or κ-casein in dilute salt solution does not necessarily exclude the caseins from a role in binding tributyrinase in milk. The occurrence of binding might require the casein to be in native form, and the ionic content of the milieu is probably important.

Clarifier-slime lipase. The lipase of molecular weight 7000 isolated from milk clarifier slime and studied by Chandan & Shahani (1963*a*,*b*), Chandan, Shahani, Hill & Scholz (1963) and Shahani & Chandan (1965) might be represented in gel-filtration elution diagrams of sodium chloride-supernatant A preparations by a small variable peak of tributyrinase activity in the region of low-molecular-weight material (Downey & Andrews, 1965*a*). However, our attempts to detect a similar peak of activity in extracts of clarifier slime, and to isolate enzyme from the slime by the method of Chandan & Shahani (1963*a*), were unsuccessful. The 22% recovery of purified lipase from clarifier slime reported by Chandan & Shahani (1963*a*) indicates that the enzyme formed a major part of the slime lipase. On the other hand, as the specific activity of lipase in clarifier slime is about three times that in whole milk on a protein basis (Nelson & Jezeski, 1955), calculations based on data given by Chandan & Shahani (1963*a*) together with a value of 30g./100ml. as the protein content of clarifier slime (Hökl & Štěpánek, 1965) show that the purified slime lipase equalled only about 0·1% of the lipase activity of the original milk. Whether or not the lipase does form an appreciable part of milk lipase is therefore uncertain. An alternative origin for it is suggested by the results of Gaffney & Harper (1965), who have found lipase in somatic cells obtained from separator slime.

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